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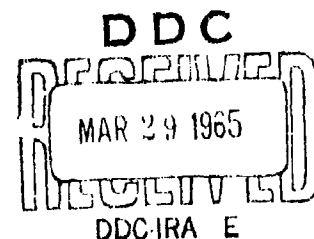
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TECHNICAL MANUSCRIPT 208

LIQUID NITROGEN PRESERVATION  
OF MAMMALIAN CELLS  
IN A CHEMICALLY DEFINED MEDIUM  
AND DIMETHYLSULFOXIDE

MARCH 1965



UNITED STATES ARMY  
BIOLOGICAL LABORATORIES  
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LIQUID NITROGEN PRESERVATION OF MAMMALIAN CELLS  
IN A CHEMICALLY DEFINED MEDIUM AND DIMETHYLSULFOXIDE

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ABSTRACT

Three established mammalian cell lines (cat kidney, L, and HeLa cells) grown in suspension in a protein-free chemically defined medium have been preserved successfully in a liquid nitrogen refrigerator in the defined medium fortified with dimethylsulfoxide. The optimal concentration of dimethylsulfoxide for the preservation of cat kidney and L cells was 4%. The optimal concentration of dimethylsulfoxide for preservation of HeLa cells was 8%. Recovery of viable cat kidney and L cells after one month's storage was 86% as judged by the trypan blue dye exclusion method. The thawed cells yielded normal growth upon inoculation into growth medium. The viability of HeLa cells after one month's storage was 86% and normal growth resulted upon reinoculation in growth medium.

## I. INTRODUCTION

In the past several years numerous freezing procedures have been described for the preservation of animal cells cultured in the presence of serum or protein hydrolyzates.

Scherer and Hoosgasian<sup>1</sup> demonstrated that HeLa and L cells frozen with ethanol and dry ice could be recovered when glycerol was used as an additive in the presence of serum. Scherer<sup>2</sup> also showed that these cells could be frozen and stored for long periods of time in the glycerol and serum combination.

Porterfield and Ashwood-Smith<sup>3</sup> showed that cells grew vigorously after being frozen in 10% dimethylsulfoxide (DMSO) plus serum. Limited growth was obtained when 10% glycerol was used in place of dimethylsulfoxide.

Dougherty,<sup>4</sup> using a lactalbumin hydrolyzate medium fortified with 15% calf serum, noted better survival with 7.5 to 12% dimethylsulfoxide than with 5 to 20% glycerol. He stated that cell survival was satisfactory only in the presence of serum. Perry<sup>5</sup> showed that 12 cell lines cultured in serum-containing media and frozen in 7.5% glycerol or 7.5% dimethylsulfoxide were successfully recultured after thawing.

Evans et al.<sup>6</sup> have shown that human skin epithelium (NCTC 3075), monkey kidney epithelium (NCTC 3526 LLCM K2), and Earle's strain L C3H mouse fibrosarcoma cells (NCTC 2071) could be successfully frozen and recovered from storage at liquid nitrogen temperatures in a serum-free medium with 6 or 12% glycerol. Their procedure required the important technique of stepwise dilution to remove glycerol from the cells.

This manuscript describes a method for preserving mammalian cells in a medium completely free of serum or protein hydrolyzate and containing dimethylsulfoxide.

## II. MATERIALS AND METHODS

Three cell lines, cat kidney, L, and HeLa, were grown in Nagle's<sup>7</sup> chemically defined medium (minus insulin)(Table 1). The cultures were incubated at 34 to 36 C in rubber-stoppered 2-liter bottles, each containing 500 ml of medium, on a New Brunswick Gyrotory shaker operating at 124 to 130 rev/min. Cell viability was determined by the trypan blue dye exclusion method. When the cell count reached approximately  $1.5 \times 10^6$  cells per ml, each culture was centrifuged at 1000 rev/min for 20 min,

TABLE 1. CHEMICALLY DEFINED MEDIUM FOR GROWTH OF CELLS  
IN SUSPENSION CULTURES

Component	Concentration, mg/liter	Component	Concentration, mg/liter
Nitrogen sources		Salts	
L-Arginine·HCl	100	NaCl	7400
L-Cysteine·HCl	75	KCl	400
L-Histidine·HCl	60	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	100
L-Isoleucine	150	NaHCO <sub>3</sub>	300
L-Leucine	300	CaCl <sub>2</sub> ·2H <sub>2</sub> O	265
L-Lysine·HCl	300	MgCl <sub>2</sub> ·6H <sub>2</sub> O	275
L-Methionine	60	Carbon sources	
L-Phenylalanine	120	Glucose	1000
L-Threonine	135	Sodium pyruvate	110
L-Tyrosine	120	Vitamins	
L-Tryptophan	60	D-Biotin	1.0
L-Valine	150	Choline·Cl	1.0
L-Glutamine	450	Folic Acid	1.0
Antibiotics, etc.		Niacinamide	1.0
Methocel 15 cps	1000	Ca pantothenate	2.0
Streptomycin	100	Pyridoxal·HCl	1.0
Phenol red	10	Thiamine·HCl	1.0
Penicillin	100,000 units/liter	i-Inositol	1.0
		Riboflavin	0.1
		B <sub>12</sub>	0.002

and the cells were resuspended in 72 ml of fresh growth medium to give a concentration of approximately ten million cells per milliliter. Twelve-ml portions of the pooled cells were then placed in six sterile, 2-oz prescription bottles and centrifuged at 1000 rev/min for 10 min. The supernatants were decanted and replaced with 12 ml of fresh medium containing DMSO in concentrations of 1 to 6% for cat kidney and L cells and 4 to 10% for HeLa cells in increments of 1%. The optimal concentration range of DMSO for satisfactory cell recovery had been determined in preliminary studies.

The cell suspension from each bottle was dispensed as 1-ml portions into 1.2-ml ampules with a 10-cc hypodermic syringe fitted with a 1½-inch, 20-gauge needle. Immediately, the ampules were pull-sealed by an automatic ampule sealer (Kahlenberg-Globe Equipment Co., Sarasota, Florida) and then placed at 4 C for 20 to 30 min.

Three ampules at each DMSO concentration were sampled for determination of zero-time viability. The remaining ampules were placed in the wire rack of the Linde liquid nitrogen freezer (BF-3, Biological Freezer) and frozen at a rate of 1 degree per min to about -40 C. The frozen samples were placed immediately in aluminum ampule racks, covered with cardboard tubes (Frozen Semen Products) and stored in the Linde liquid nitrogen refrigerator (LNR-35) at -196 C.

The viability of frozen cells was determined by removing three ampules for each sampling period and thawing immediately in a 40 C agitating water bath. Suspensions were completely thawed in 30 to 55 seconds. The ampules were opened aseptically by scoring the neck with a file and snapping off the top. The thawed cell suspensions were aseptically transferred from the ampule to a 12-ml conical centrifuge tube containing 3 ml of fresh growth medium. The suspended cells were then centrifuged for about 10 min at approximately 1000 rev/min.

This process was repeated twice and after the second washing the cells were resuspended in 25 ml of growth medium in a 100-ml serum bottle and the viability was determined. Cell suspensions were then placed on the shaker, the cells were counted daily, and the medium was changed at various intervals until a maximum cell population was reached.

### III. RESULTS

The data presented in Fig. 1 show that L cells, continuously cultured in a serum-free system and frozen and stored as described, gave the highest recovery of viable cells, 90, 88, and 86%, in medium containing 4% DMSO.

However, 3, 5, and 6% DMSO also provided satisfactory recoveries of 80, 81, and 84% after storage for one month.

Cells frozen in 1% DMSO gave evidence of survival but failed to grow after reincubation in fresh medium. The trypan blue dye exclusion method indicated that all cells were dead after 3 days' incubation. Cells frozen without DMSO appeared to be dead immediately after thawing.

The data presented in Fig. 2 show the effect of the defined medium and DMSO on survival of HeLa cells.

As shown, 8% DMSO provided the most consistent viability values (87, 81, and 86%) after storage.

Fig. 3 shows the effect of storage conditions on cat kidney cells. With this cell line, 4% DMSO showed the best viability (74, 81, and 86%) throughout the storage period.

Fig. 4 shows growth curves of L, HeLa, and cat kidney cells after storage in 4, 8, and 4% DMSO, respectively, for one month. The peak growth yields of these cells were  $51.0 \times 10^5$ ,  $47.0 \times 10^5$ , and  $41.0 \times 10^5$  cells per ml respectively. These growth curves compare favorably with those obtained with unfrozen controls.

These studies indicate that although L and cat kidney cells frozen in 4% DMSO and HeLa cells frozen in 8% DMSO gave higher recovery than was observed with the other concentrations of DMSO, they were nevertheless able to grow well after storage at the levels of DMSO near these optimum concentrations. It was interesting to note that the epithelial-like HeLa cells required higher concentrations of DMSO for optimal recovery than did the fibroblast-like L and cat kidney cells. Further studies are indicated to determine if this is a general phenomenon.

In summary, we have shown that it is possible to grow and preserve mammalian cells suspended in a simple chemically defined medium with DMSO as an additive in the complete absence of serum or serum products. The typical growth curves obtained from stored samples indicate that the conditions employed have not altered this important characteristic.

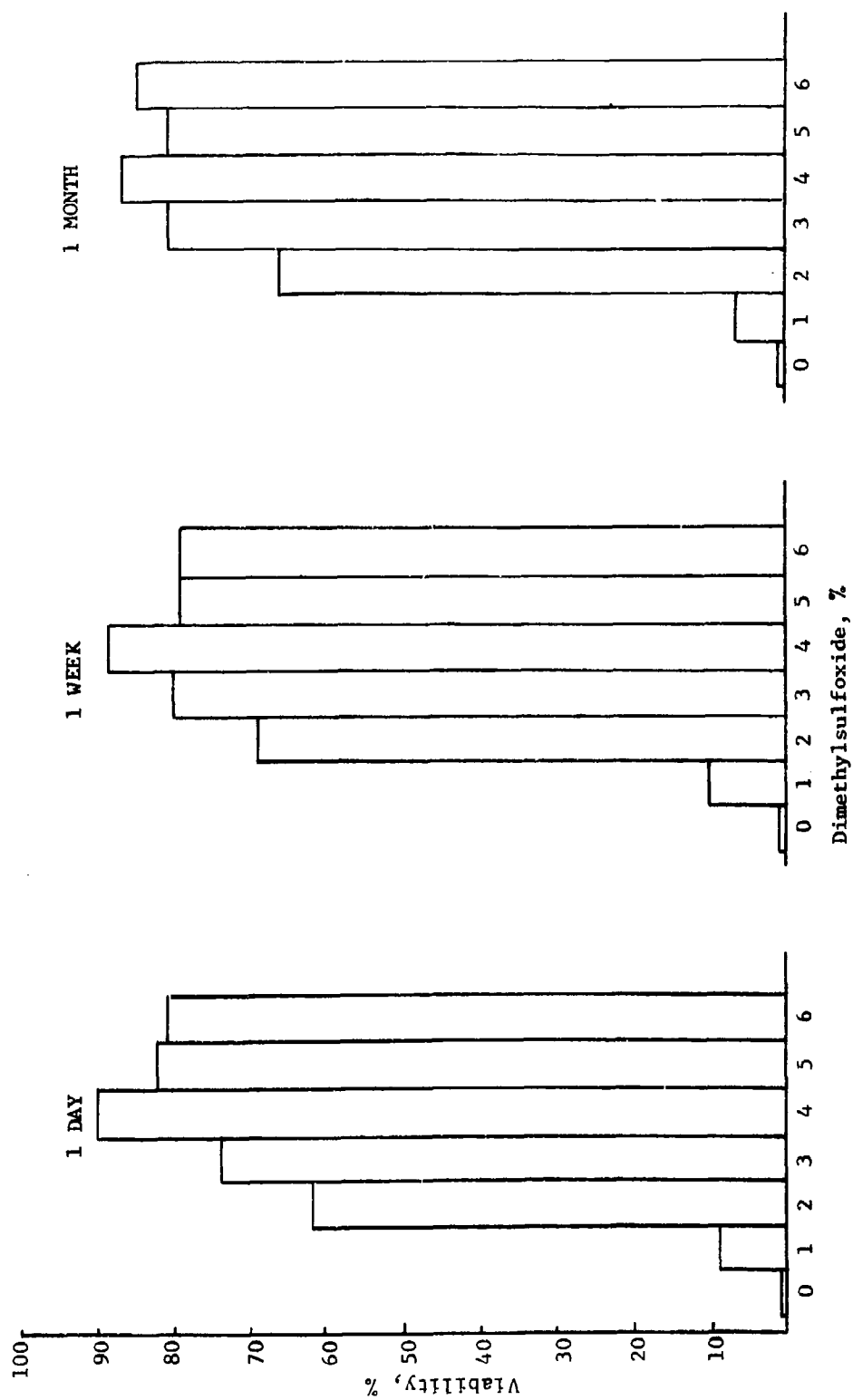


Figure 1. Viability of L Cells Frozen and Stored in a Chemically Defined Medium Containing DMSO.

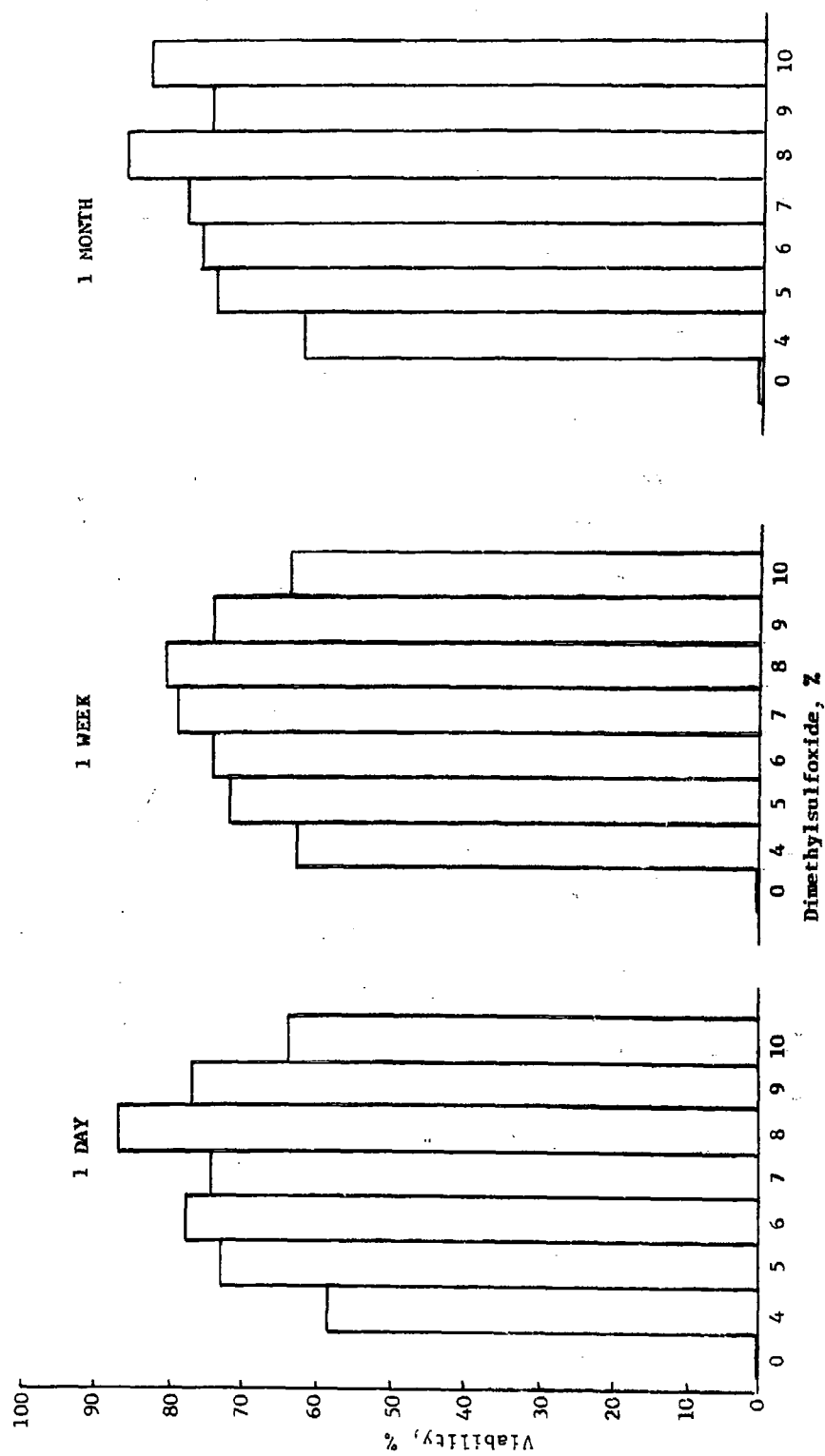


Figure 2. Viability of HeLa Cells Frozen and Stored in a Chemically Defined Medium Containing DMSO.

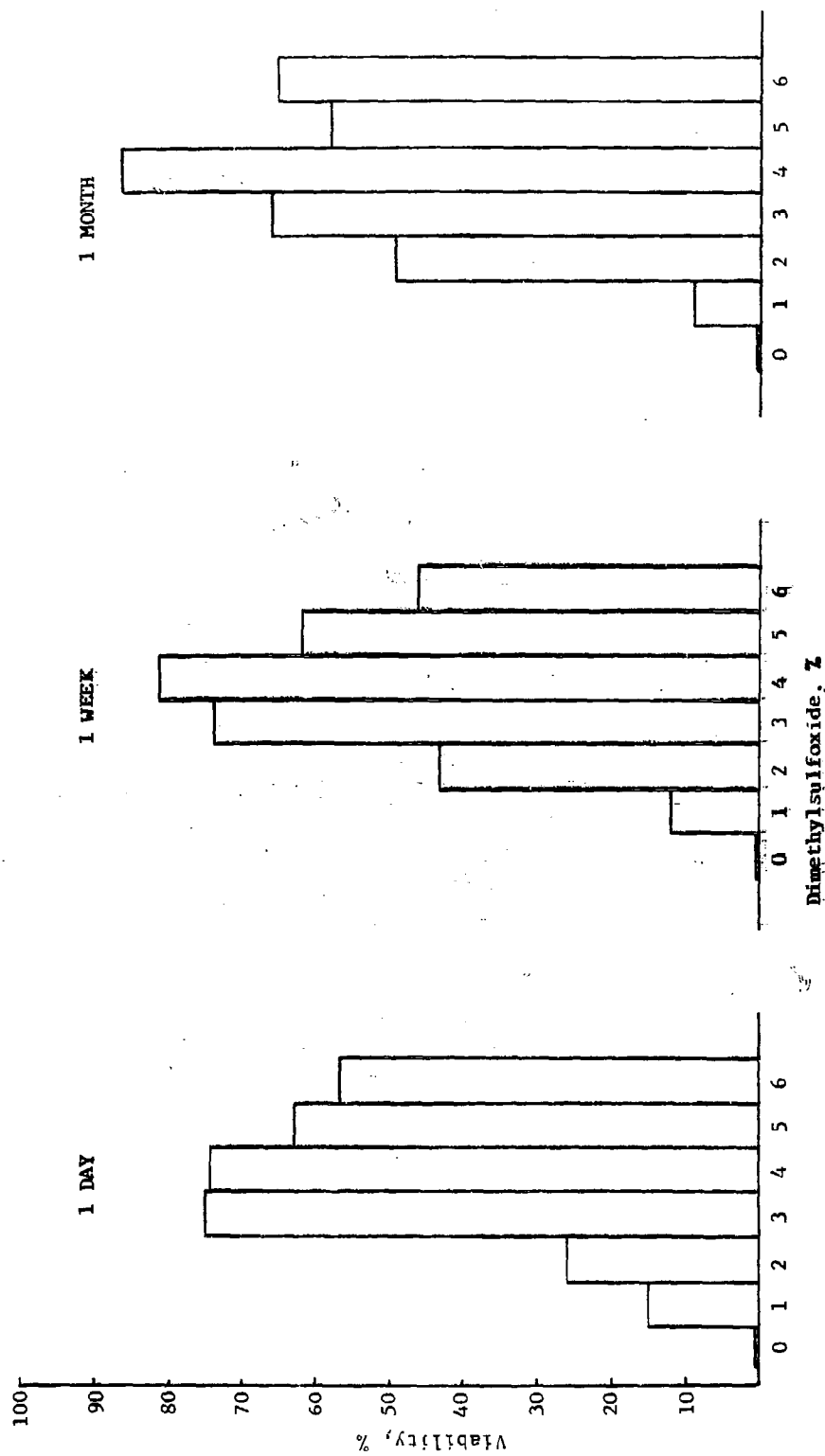


Figure 3. Viability of Cat Kidney Cells Frozen and Stored in a Chemically Defined Medium Containing DMSO.

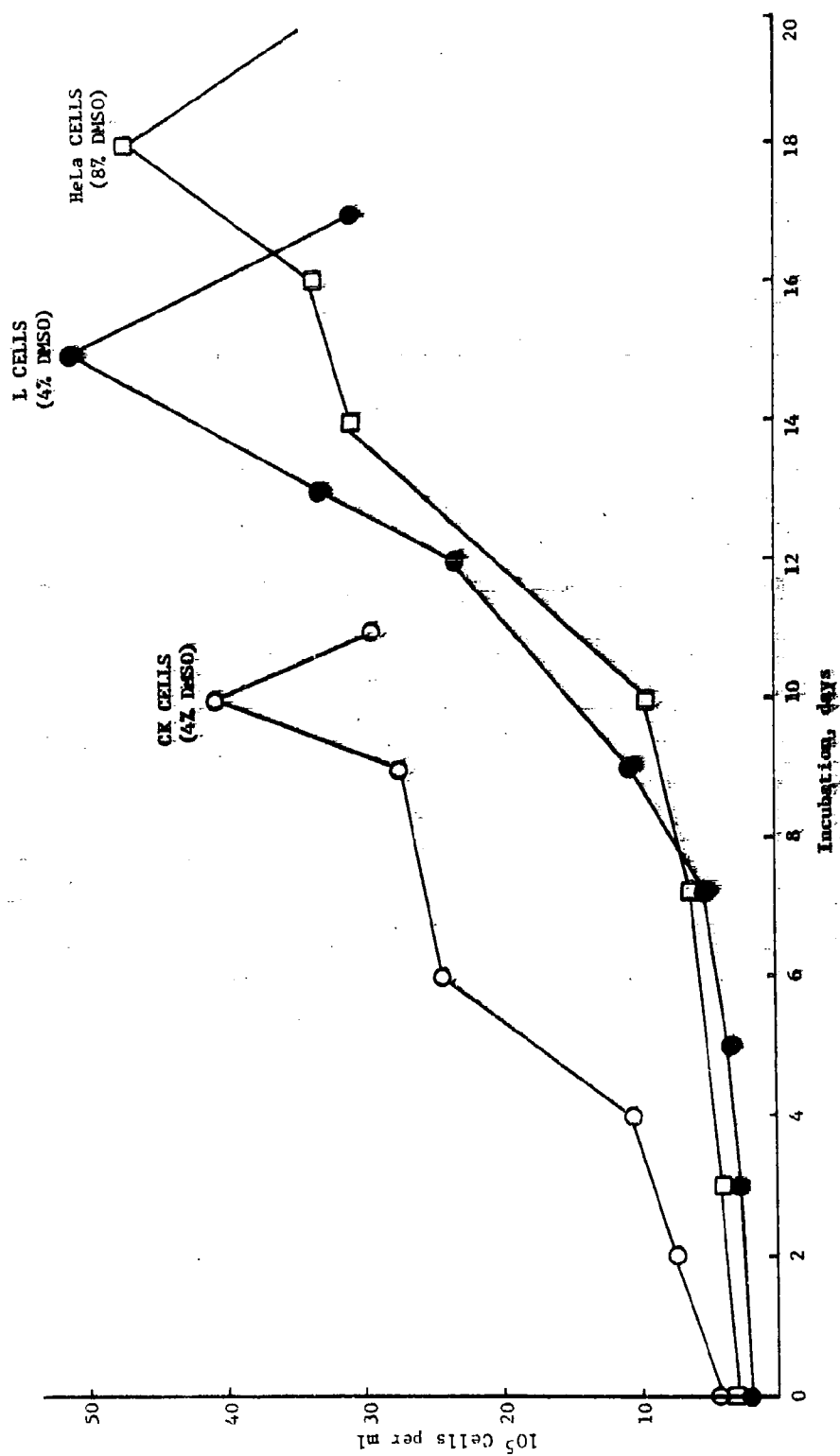


Figure 4. Growth of L, HeLa, and Cat Kidney Cells after Storage in Liquid Nitrogen at Optimum Concentration of DMSO for One Month.

LITERATURE CITED

1. Scherer, W.F., and A.C. Hoosgasian. 1954. Preservation at subzero temperatures of mouse fibroblasts (strain L) and human epithelial cells (strain HeLa). *Proc. Soc. Exp. Biol. Med.* 87:480-487.
2. Scherer, W.F. 1960. Effects of freezing speed and glycerol diluent on 4-5 year survival of HeLa and L cells. *Exp. Cell Res.* 19:175-176.
3. Porterfield, J.S., and M.J. Ashwood-Smith. 1962. Preservation of cells in tissue culture by glycerol and dimethyl sulfoxide. *Nature* 193:548-550.
4. Dougherty, R.M. 1962. Use of dimethyl sulfoxide for preservation of tissue culture cells by freezing. *Nature* 193:550-552.
5. Perry, V.P. 1963. Techniques of viable tissue storage. *Federation Proc.* 22:102-108.
6. Evans, V.J., H. Montes de Oca, J.C. Bryant, E.L. Schilling, and J.E. Shannon. 1962. Recovery from liquid nitrogen temperature of established cell lines frozen in chemically defined medium. *J. Nat. Cancer Inst.* 29:749-757.
7. Nagle, S.C., Jr., H.R. Tribble, Jr., R.E. Anderson, and N.D. Gary. 1963. A chemically defined medium for the growth of animal cells in suspension. *Proc. Soc. Exp. Biol. Med.* 112:340-344.

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